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Cholinergic imbalance in lumbar spinal cord of a rat model of multiple sclerosis

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Abstract

Cholinergic dysfunction in the central nervous system is an important characteristic of multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). By using a rat EAE model, upregulation of vesicular acetylcholine transporter (VACHT) level in the EAE rat lumbar spinal cord was detected by western blot and immunostaining, and was associated with lymphocyte filtration and glial activation. *Ex vivo* and *in vitro* autoradiography studies with [¹⁸F]VAT, a VACHT-specific radioligand, also revealed increased tracer uptake in EAE rat lumbar spinal cord compared with shams. These studies on VACHT expression suggest central cholinergic imbalance during EAE progression.

Graphical Abstract

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Conflict of interest

The authors declare that they have no competing of interests.

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Keywords

Vesicular acetylcholine transporter; Experimental autoimmune encephalomyelitis; Neuroinflammation

1. Introduction

Multiple sclerosis (MS) is the most common demyelinating disease of the central nervous system (CNS) and the leading cause of non-traumatic neurological disability among young adults (Karussis, 2014). The pathology of MS lesions is characterized by demyelination, inflammation and axon/neuronal pathology (Mahad et al., 2015). Cholinergic dysfunction is commonly observed in the CNS of MS patients and animal models of experimental autoimmune encephalomyelitis (EAE) (Tata et al., 2014, Vogt et al., 2009). Lower motor neurons, which are cholinergic neurons and located in the ventral horn of the spinal cord, undergoes substantial loss ranging from 15% to 48% in MS cases compared to healthy controls (Vogt et al., 2009). In MS brain, activity and protein expression of choline-acetyltransferase (ChAT) is also decreased in hippocampus (Kooi et al., 2011). Meanwhile, evidence that cholinergic modulation of inflammatory response contributes to MS progression is accumulating. The ChAT protein level in the cerebrospinal fluid of MS patients is more than two-fold higher compared to controls (Vijayaraghavan et al., 2013). Moreover, activation of $\alpha 7$ nicotinic acetylcholine receptors (nAChRs) by nicotine suppresses production of pro-inflammatory cytokine, infiltration of proinflammatory monocytes and neutrophils into the CNS, and alleviates the severity of EAE (Jiang et al., 2016, Nizri et al., 2009, Reale et al., 2015). While cholinergic imbalance in the course of MS is complex and reflects the heterogeneity of MS pathophysiology, it provides important information for evaluation of disease severity of MS and potentially could be a therapeutic target for MS.

Vesicular acetylcholine transporter (VACHT) which loads acetylcholine (ACh) into vesicles, is a reliable biomarker for cholinergic system and has been investigated extensively in cognitive dysfunction (Efange, 2000). A single photon emission computed tomography (SPECT) study using a specific VACHT radioligand [^{123}I]IBVM demonstrated early cholinergic degeneration occurs in the early stage of Alzheimer's disease (AD) (Mazere et al., 2008). As the disease progresses in AD patients, the levels of VACHT change in a similar fashion and magnitude to other cholinergic marker proteins, in particular ChAT (Kuhl et al., 1999, Kuhl et al., 1996). Recent studies revealed that VACHT may participate in the pathogenesis of inflammatory response. Rodents with acute lung inflammation showed reduced VACHT expression (Lips et al., 2007). On the other hand, transgenic mice with reduced VACHT levels (VACHT KD^{hom} mice) show airway inflammation with enhanced inflammatory markers, hyper-responsiveness and increased collagen and elastic fiber deposition, indicating cholinergic deficiency impair lung function and produce local inflammation (Pinheiro et al., 2015). VACHT KD^{hom} mice also developed severe kidney disease with a prominent inflammatory response and high expression of pro-inflammatory cytokines (Silva et al., 2016). Moreover, the decreased ability to release ACh in VACHT knock down mice exacerbates systemic and cerebral inflammation and are more susceptible

to inflammation and sickness behavior induced by lipopolysaccharide (LPS) (Leite et al., 2016). Very recently, cholinergic PET imaging using [^{11}C]donepezil (an AChE radioligand) and [^{18}F]FEOBV (an VACHT radioligand) shows distinct accumulation of both tracers in a murine abscess model (Jorgensen et al., 2017).

Accordingly, we performed a pilot study to investigate the change of VACHT protein expression in rat lumbar spinal cord in response to demyelination and neuroinflammation induced by EAE, using western blot and immunostaining. In addition, we further performed autoradiography studies to assess the change of VACHT expression in EAE rat spinal cord. The promising VACHT radiotracer, (-)-(1-(8-(2-[^{18}F]fluoroethoxy)-3-hydroxy-1,2,3,4-tetrahydronaphthalen-2-yl)-piperidin-4-yl)(4-fluorophenyl)-methanone ([^{18}F]VAT, Figure 5), possessing high potency ($K_i = 0.59 \text{ nM}$), high selectivity (>10,000-fold selectivity for VACHT versus sigma receptors), and favorable *in vivo* binding profile (Jin et al., 2015, Tu et al., 2015), was utilized for autoradiography studies.

2. Methods

2.1. EAE rat model

All animal experiments were conducted in compliance with the Guidelines for the Care and Use of Research Animals under protocols approved by Washington University's Institutional Animal Care and Use Committee. The EAE rat model was established as previous reported (Liu et al., 2016). In brief, female Lewis rats (Charles River Laboratories, Inc., Wilmington, MA), weighing 100–125 g, were used for EAE induction. Before each immunization, a myelin basic protein (MBP) emulsion was freshly prepared using a MBP fragment (MBP68-86, AnaSpec Inc., San Jose, CA) and complete Freund's adjuvant (CFA), containing 1 mg/mL heat-inactivated *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI). Immunization of rats was performed under anesthesia (2–3% isoflurane in O_2), by injecting 200 μL of emulsion, divided equally between the hind footpads. Control rats were injected with an identical volume of a PBS/CFA emulsion.

Body weight and neurological symptoms were checked daily on the animal post-injection of MBP. Neurological impairment was graded as follows: 0, no symptoms; 1, flaccid tail; 2, hindlimb weakness; 3, paraparesis; 3.5, unilateral hindlimb paralysis; 4, bilateral hindlimbs paralysis; and 5, bilateral hindlimbs paralysis and incontinence. Rats with score ≥ 4.0 in EAE group were euthanized and processed for western blot ($n = 4$), immunohistological studies ($n = 4$), as well as *in vitro* autoradiography ($n = 2$). Lumbar spinal cords were rapidly dissected on ice. Isolated tissues were then dissected and cut in half. One half of the lumbar spinal cord was fixed in 10% formalin and processed for immunohistological studies. The other half was snap frozen and used for Western blot and *in vitro* autoradiographic studies. Additional groups of EAE and sham rats were used for *ex vivo* autoradiography ($n = 4$). The total number of rats that used in this study was 28, $n = 14$ for EAE and sham groups.

2.2. Western blot quantification of VACHT protein expression

Rats were euthanized and lumbar spinal cords were frozen for western blot. Frozen tissues were homogenized in cold RIPA buffer containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl,

1% NP-40, 1% sodium deoxycholate, 0.1% SDS with protease inhibitor cocktails, and centrifuged at 12,000 g for 20 min at 4 °C to remove the insoluble material. The pellets were discarded and supernatant was further mixed with the loading buffer containing 65.8 mM Tris-HCl, pH 6.8, 2.1% w/v SDS, 26.3% v/v glycerol, 0.01% w/v bromophenol blue and boiled at 95 °C for 5–7 min. The prepared samples were electrophoresed on 12% SDS-PAGE, before transfer to polyvinylidene difluoride (PVDF) membrane and blocked with 5% nonfat dry milk for 1h at room temperature. After washing, the membrane was incubated with a mouse anti-rat VACHT antibody (1:500, Abcam, Cambridge, MA) for 16 h at 4 °C followed by incubation with horseradish peroxidase (HRP) linked secondary antibody (1:10,000, R&D systems, Minneapolis, MN) at room temperature for 1h. After the incubation, blots were washed and developed using Immobilon Western chemiluminescent HRP substrate (Thermo scientific, Pittsburgh, PA) following the recommended procedure. To normalize the protein bands to gel loading control, β actin was probed as an internal control. A digital gel image J 1.49V (NIH, Bethesda, MD) analysis software was used for semi-quantification of VACHT immunoreactivity.

2.3. Histological analysis

To determine the VACHT expression changes in EAE rat spinal cord, immunohistochemistry was carried out. Hematoxylin and eosin (H&E) staining was performed using a routine procedure to visually check lesion morphology surrounding spinal cord lesions. Sections were deparaffinized in xylene and rehydrated through a graded alcohol series to water, followed by antigen retrieval. Endogenous peroxidase activity was quenched with a solution of 3 % H₂O₂ in PBS for 15 min. Sections were incubated in blocking buffer for 15 min before incubating with the mouse anti -rat VACHT antibody (1:100, Abcam, Cambridge, MA) overnight at 4 °C. Primary anti body binding was detected using an anti–mouse HRP-DAB staining kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

To further identify the cellular location of VACHT expression, immunofluorescence was performed on deparaffinized and hydrated slides. The primary antibodies were added as a mixture and incubated with the sections at 4 °C overnight. The secondary fluorescently labeled antibodies were then added and incubated in the dark for 60 min. The primary antibodies and secondary antibodies used in this study were: 1) the mouse anti-rat VACHT antibody (1:100) with a fluorescein-conjugated goat anti-mouse IgG antibody (1:100, Jackson ImmunoResearch Lab, West Grove, PA); 2) a rabbit anti -rat CD19 antibody (1:100, Abnova, Taipei, Taiwan) or a rabbit anti-rat CD3 antibody (1:100, Abcam, Cambridge, MA) with a Dylight 594 Horse anti-rabbit antibody (1:100, Vector Laboratories, Burlingame, CA); 3) a goat anti-rat VACHT antibody (1:500, Millipore, Billerica, MA) with a Dylight 488 horse anti-goat antibody (1:100, Vector Laboratories, Burlingame, CA); 4) a mouse anti-rat GFAP antibody (1:400, Millipore, Billerica, MA) or a mouse anti-rat Iba-1 antibody (1:300, Millipore, Billerica, MA) with a Dylight 594 horse anti-mouse antibody (1:100, Vector Laboratories, Burlingame, CA). The sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and cover-slipped. A Nikon E600 microscope with a charge-coupled device camera was used to obtain all photomicrographs.

2.4. Radiosynthesis of [^{18}F]VAT

The radioligand [^{18}F]VAT was produced as reported recently by our group (Tu et al., 2015). The radiochemical yield was 40–50% (decay corrected to end of bombardment (EOB) with a radiochemical purity > 99%, the chemical purity of > 95%, and the mean specific activity was > 63 GBq/ μmol (decay corrected to EOB).

2.5. *Ex vivo* autoradiography

In order to explore the potential application of the promising VACHT radioligand [^{18}F]VAT in assessing VACHT expression change, *ex vivo* autoradiography studies were performed. Animals received a bolus injection of [^{18}F]VAT (~0.5 mCi) via the tail vein under ~2 % isoflurane anesthesia and euthanized 60 min post injection of tracer. Lumbar spinal cords were harvested, embedded in optimal cutting temperature (OCT) compound (Fisher healthcare, Houston, TX) and frozen on dry ice. Sections (100 μm) were prepared using a cryostat and exposed to a storage phosphor screen (BAS-IP-MS-2025) in an imaging plate overnight in -80°C in the dark. The distribution of radioactivity were visualized by a Fuji Bio-Imaging Analyzer FLA-7000 (Fuji Photo Film, Tokyo, Japan). Photostimulated luminescence was quantified using Multi Gauge v3.0 software. Data were background - corrected and expressed as photo-stimulated luminescence signals per square millimeter (PSL/ mm^2).

2.6. *In vitro* autoradiography

To exclude the possibility that the increase of tracer uptake was caused by the leakage of blood-brain barrier (BBB), 20 μm slices of snap-frozen rat lumbar spinal cord were incubated with 1 nM [^{18}F]VAT in the assay buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , pH 7.4) at room temperature for 60 min. Non-specific binding was determined by using adjacent sections incubated with an additional 10 μM vesamicol, a potent VACHT Inhibitor, in the assay buffer. Following the incubation, the sections were washed and exposed to the storage phosphor screen in an imaging cassette overnight in -80°C in the dark. The distribution of radioactivity were visualized by a Fuji Bio-Imaging Analyzer FLA-7000 (Fuji Photo Film, Tokyo, Japan).

2.7. Statistical Analysis

All experimental data are expressed as means $\pm$ standard deviation (SD). Data generated from Western blot and [^{18}F]VAT autoradiography were analyzed by Student's *t* test and $P < 0.05$ is considered statistically significant.

3. Results

3.1. Western blot and immunohistochemistry

The VACHT protein level in lumbar spinal cord of both sham and EAE rats were measured by western blot. As shown in Fig. 1A&B, the protein level of VACHT of lumbar spinal cord increased ~43.0% in the EAE rats compared to sham rats (VACHT/ β -actin ratio: 8.06 ± 0.21 v.s. 5.62 ± 0.13 , $n = 4$, $P < 0.01$).

H&E staining revealed severe infiltration of inflammatory cells in both gray matter and white matter in EAE rat lumbar spinal cord. Modest loss of the lower motor neurons was also observed in the EAE rats. VACHT-positive cells was observed within both gray matter and white matter of rat spinal cord by immunohistochemical staining (Fig. 1C). Lower motor neurons in the ventral horn within gray matter were VACHT-positive, and the VACHT expression was reduced in EAE rats compared to sham. In contrast, VACHT-positive infiltrated inflammatory cells were found throughout the lumbar spinal cord of EAE rats, but not in sham.

3.2. Immunofluorescence

Furthermore, immunofluorescence staining studies revealed the co-localization of VACHT and T cell biomarker CD3 or B cell biomarker CD19 (Fig. 2). Iba-1 (the biomarker for microglia) and GFAP (the biomarker for astrocytes) were also co-localized with VACHT expression in EAE rat lumbar spinal cord, but not in sham (Fig. 3&4). The immunofluorescence data demonstrated both infiltrated lymphocytes and activated glial cells contribute to upregulated expression of VACHT in response to inflammation in EAE rat lumbar spinal cord.

3.3. *Ex vivo* and *in vitro* autoradiography

Our *ex vivo* autoradiography suggested that the uptake of [¹⁸F]VAT in the lumbar spinal cord (581.26 ± 165.88 PSL/mm²) of EAE rats was consistently higher than that in sham rats (465.10 ± 136.09 PSL/mm²), with a percentage of approximately 25% ($n = 4$, $P = 0.015$, Fig. 5A&B). Our *in vitro* autoradiographic studies also displayed similar trend of tracer uptake (Fig. 5C). Blocking studies with a VACHT ligand vesamicol decreased the signal by ~50%, demonstrating that the tracer uptake mainly resulted from the binding of [¹⁸F]VAT to VACHT in rat lumbar spinal cord.

4. Discussion

In the current study, western blot and immunohistological analysis revealed the increase of VACHT level in the EAE rat lumbar spinal cord compared to sham, and further identified infiltrated lymphocytes and activated glial cells as cellular resources of upregulated VACHT expression. In addition, autoradiography with the specific VACHT radioligand [¹⁸F]VAT successfully detected the change of VACHT expression.

The MBP-induced EAE rat model was used in this study as a model of neuroinflammation, which is characterized by severe CNS inflammation, with acute and modest demyelination (without remyelination) (Constantinescu et al., 2011, Meeson et al., 1994). The most severe inflamed lesion in this model is located inside the lumbar spinal cord, demonstrated by H&E staining. Notably, H&E staining revealed severe inflammatory cell infiltration, and only modest lower motor neuron loss in the grey matter, suggesting cholinergic imbalance in lumbar spinal cord of this EAE rat model is majorly resulted from inflammation instead of neuron loss.

Western blot was used to qualify the difference of VACHT expression in rat lumbar spinal cord between EAE and sham. Similar to a previous research (Tomassoni et al., 2012), our

results revealed multiple bands, which may be resulted from the mature, post-translationally modified form of VACHT protein. The band with a lowest molecular weight in EAE group may represent a degradation products (Tomassoni et al., 2012), which is absent in sham. Immunohistochemical analysis of VACHT expression further revealed modest decrease in lower motor neurons and dramatic increase in inflammatory cells. It is inferred that the decrease of neuron-derived VACHT expression is overcome by the increase of VACHT expressed by inflammatory cells, resulting in the upregulation of total VACHT protein level and increased uptake of [^{18}F]VAT in EAE rat spinal cord.

Autoimmune inflammatory damage of multiple sclerosis is mainly mediated by lymphocytes ; lymphocyte (mainly T cells and B cells) infiltration correlated with the activity of demyelinating lesions (Frischer et al., 2009). It was demonstrated that T cells and B cells could be cholinergic, and may regulate local innate immunity (Kawashima et al., 2015, Kawashima et al., 2007, Reardon et al., 2013). However, it is still not clear whether T cells and B cells express VACHT (Ogawa et al., 2003, Tayebati et al., 2002). In the current study, immunofluorescence with VACHT, T cell specific marker CD3 and B cell specific marker CD19 were used to discriminate VACHT-positive infiltrated lymphocytes in lumbar spinal cord of EAE rats. The co-localization study revealed a large number of T cells and B cells with positive VACHT expression. The VACHT-positive T cells and B cells distribute throughout gray matter and white matter of EAE rat lumbar spinal cord.

Glial cell activation is also prevalent during inflammatory progression in MS (Correale, 2014, Correale and Farez, 2015). Previous study showed ChAT is secreted by cultured human-brain astrocytes, and could be upregulated in response to the stimulation of CD3 or lipopolysaccharides (LPS) (Vijayaraghavan et al., 2013). However, there is no evidence of VACHT expression in microglia and astrocytes. Therefore, we examined the co-localization of VACHT and Iba-1/GFAP (biomarkers for microglia and astrocytes) by immunofluorescence. A large amount of activated microglia and astrocytes in EAE rat spinal cord showed positive VACHT expression, while no VACHT signal was observed in both microglia and astrocytes at rest in sham. Thus activated glial cells is one of the sources of VACHT expression in EAE lumbar spinal cord.

To detect cholinergic imbalance in EAE rat spinal cord by the VACHT-specific radioligand [^{18}F]VAT, *ex vivo* autoradiography was utilized in the current study, and revealed a high tracer uptake in rat spinal cord. To exclude the possibility that the increase of tracer uptake was caused by the leakage of blood-brain barrier (BBB), another group of rats were used for *in vitro* autoradiography and the result was consistent with *ex vivo* autoradiography. The consistency of *in vitro* and *ex vivo* data suggested that higher uptake of [^{18}F]VAT in EAE rat spinal cord is attributed to the upregulation of VACHT expression, instead of BBB breakdown. Based on the promising autoradiography data, initial microPET studies were conducted. Due to relatively high spine bone uptake, the attempt to visualize rat spinal cord via [^{18}F]VAT microPET turned out to be very challenging (Data not shown). In agreement with our finding, extensive expression of VACHT RNA and protein was reported in maxilla, the upper jawbone of rat (Guo et al., 2014). On the other hand, our previous biodistribution study of [^{18}F]VAT in normal rats showed stable bone uptake of the radioactivity with 0.40–0.55 %ID/g (injection dose %/gram) from 5 min to 120 min post-injection (Tu et al., 2015).

The relative stable lower bone uptake of [^{18}F]VAT suggested that *in vivo* defluorination of [^{18}F]VAT in rats was negligible. In addition, our human PET imaging study of [^{18}F]VAT in the brain also indicated that *in vivo* defluorination of [^{18}F]VAT in human was inconsiderable. Compared to the inflamed spinal cord uptake of [^{18}F]VAT, the relatively high bone uptake of [^{18}F]VAT could be largely attributed to extensively expressed VAcHT in rat spine bone. Distant CNS regions from bone tissue may be chosen for further investigation of [^{18}F]VAT in imaging neuroinflammation to avoid overshadowing. On the other hand, relative high uptake of [^{18}F]VAT in bone tissue indicates potential application in imaging cholinergic signaling by [^{18}F]VAT PET during bone formation/remodeling or under abnormal conditions (Che et al., 2016).

In conclusion, the increase of VAcHT level in the EAE rat lumbar spinal cord was founded by western blot and immunohistochemistry. Immunofluorescence staining further revealed that upregulated VAcHT expression was co-localized within infiltrated lymphocytes and activated glial cells. In addition, the change of VAcHT expression in EAE rat spinal cord was also successfully detected by autoradiography studies using a VAcHT-specific radioligand [^{18}F]VAT. However, the relative high spine bone uptake limits the implementation of PET with [^{18}F]VAT in MS via quantification of VAcHT expression change in inflamed spinal cord *in vivo*.

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List of abbreviations

ACh	acetylcholine
AD	Alzheimer’s disease
BBB	blood-brain barrier
CFA	complete Freund’s adjuvant
ChAT	choline-acetyltransferase
CNS	central nervous system
EAE	experimental autoimmune encephalomyelitis
H&E	Hematoxylin and eosin
HRP	horseradish peroxidase
IHC	immunohistochemistry

IF	immunofluorescence
LPS	lipopolysaccharide
MAP	maximum a posteriori
MBP	myelin basic protein
MS	multiple sclerosis
nAChR	nicotinic acetylcholine receptor
NHP	nonhuman primate
OCT	optimal cutting temperature
PBS	phosphate-buffered saline
PSL/mm²	photo-stimulated luminescence signals per square millimeter
PVDF	polyvinylidene difluoride
SPECT	single photon emission computed tomography
VACht	vesicular acetylcholine transporter

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Highlights

- Central cholinergic dysfunction in EAE rats was investigated.
- Upregulated VACHT protein level in EAE rat spinal cord was detected.
- Increased VACHT expression was consistent with tracer uptake of [^{18}F]VAT.
- The increase was also associated with lymphocyte filtration and glial activation.

Fig. 1.

Quantitative analysis of VAcHT protein expression in rat spinal cord. A) Representative western blot bands of VAcHT and β -actin. B) Normalization of VAcHT expression by β -actin revealed the protein level of VAcHT of lumbar spinal cord increased 43.36% in the EAE rats compared to sham rats ($n = 4$, $P < 0.01$). C) H&E staining in the upper panel showing severe infiltration of inflammatory cells and modest lower motor neuron loss in EAE rat lumbar spinal cord. Immunohistochemical staining in the lower panel revealed less VAcHT expression on lower motor neurons in EAE rats compared to sham. In contrast, VAcHT-positive infiltrated inflammatory cells were found throughout the lumbar spinal cord of EAE rats, while they were absent in sham. Inflammatory cells are indicated by red/white arrows, neurons are indicated by black arrows.

Fig. 2.

Immunofluorescence staining using VACHT and biomarkers for T, B lymphocytes in EAE rat spinal cord. The upper panels showing co-localization of VACHT and CD3 (the biomarker for T lymphocytes), indicating by white arrows; the lower panels showing co-localization of VACHT and CD19 (the biomarker for B lymphocytes), indicating by white arrows.

Fig. 3.

Immunofluorescence staining using VACHT and biomarkers for microglia and astrocytes in EAE rat spinal cord. The upper panels showing co-localization of VACHT and Iba-1 (the biomarker for microglia), indicating by white arrows; the lower panels showing co-localization of VACHT and GFAP (the biomarker for astrocytes), indicating by white arrows.

Fig. 4. Immunofluorescence staining using VACHT and biomarkers for microglia and astrocytes in sham rat spinal cord. No co-localization was observed for VACHT and Iba-1, or VACHT and GFAP.

Fig. 5. *Ex vivo* and *in vitro* autoradiography of [¹⁸F]VAT binding in rat lumbar spinal cord. A) Representative *ex vivo* autoradiographic images showing higher tracer uptake in EAE tissues than sham. B) Quantitative analysis of *ex vivo* autoradiography. C) Representative *in vitro* autoradiographic images with and without blocking by 10 μ M vesamicol. The structure of [¹⁸F]VAT was shown on the right top.